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Identification of influenza A pandemic (H1N1) 2009 variants during the first 2009 influenza outbreak in Mexico City

Hector M. Zepeda[a,*], Lizbeth Perea-Araujo[a], Paola B. Zarate-Segura[b], Joel A. Vázquez-Pérez[a], Ángel Miliar-García[c], Claudio Garibay-Orijel[b], Aarón Domínguez-López[c], Jesús A. Badillo-Corona[d], Eduardo López-Orduña[e], Octavio P. García-González[f], Ignacio Villaseñor-Ruíz[g], Armando Ahued-Ortega[g], Leopoldo Aguilar-Faisal[a], Jorge Bravo[a], Eleazar Lara-Padilla[h], Ricardo J. García-Cavazos[h]

[a] *Laboratorio de Medicina de Conservación, Escuela Superior de Medicina, Instituto Politécnico Nacional, Plan de San Luis y Díaz Mirón s/n, Col. Casco de Santo, Mexico City 11340, Mexico*
[b] *Laboratorio de Bioingenieria, Departamento de Bioingenieria, Unidad Profesional Interdisciplinaria de Biotecnología, Instituto Politécnico Nacional, Mexico City 07340, Mexico*
[c] *Laboratorio de Biología Molecular, Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico City 11340, Mexico*
[d] *Departamento de Bioprocesos, Unidad Profesional Interdisciplinaria de Biotecnología, Instituto Politécnico Nacional, Mexico City 07340, Mexico*
[e] *Amplibio, Mexico City 03700, Mexico*
[f] *Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Mexico State 54090, Mexico*
[g] *Secretaria de Salud del Distrito Federal, Mexico City 06820, Mexico*
[h] *Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico City 11340, Mexico*

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ABSTRACT

Background: In March 2009, public health surveillance detected increased numbers of influenza-like illness presenting to hospitals in Mexico City. The aetiological agent was subsequently determined to be a novel influenza A (H1N1) triple reassortant, which has spread worldwide. As a consequence the World Health Organisation has declared the first Influenza pandemic of the 21st century.
Objectives: To describe clinically and molecularly the first outbreak of influenza A pH1N1 (2009) during 1–5 May to establish a baseline of epidemiological data for pH1N1. Also, to monitor for the emergence of antiviral resistance, and mutations affecting virulence and transmissibility.
Study design: Samples were collected from 751 patients with influenza-like symptoms throughout Mexico City and were tested for influenza A pH1N1 (2009) using real-time PCR. In the samples that were positive for influenza A pH1N1 (2009) fragments from the haemagglutinin (H1) and neuraminidase (N1) genes were sequenced.
Results: A total of 203/751 (27%) patients were positive for the pandemic H1N1 (2009) virus (53% male and 47% female). The 0–12-year-old group was the most affected 85/751 (42%). Sequence analysis showed five new variants of the pandemic H1N1 (2009) virus for NA: G249E (GQ292900), M269I (GQ292892), Y274H (GQ292913), T332A (GQ292933), N344K (GQ292882), and four variants for HA: N461K (GQ293006), K505R (GQ292989), I435V (GQ292995), I527N (GQ292997).
Conclusions: We have provided a baseline of epidemiological data from the first outbreak of influenza A pH1N1 (2009) during 1–5 May in Mexico City. The sequencing of partial fragments of the HA and NA genes did not show the presence of previously described mutations affecting known sites of antiviral resistance in seasonal influenza A such as the H275Y (oseltamivir resistance), R293 or N295 etc.

1. Background

From early March 2009, uncommon cases of atypical pneumonia started to appear in Mexico City's hospitals.[1] During April, the number of cases increased and spread to almost all boroughs in the city.[2] On June 11 the World Health Organization (WHO) decided to raise the level of influenza pandemic alert from phase 5 to phase 6, and since then, more than 100 countries have officially reported more than 7 million cases of infection with the influenza A pH1N1 (2009) virus, from which at least 13500 patients have died.[3] During the initial outbreak, Mexico reported most of the infection and the highest number of casualties around the world.[1] Early studies found that this novel influenza virus contained the hemagglutinin (HA), the nucleoprotein (NP) and the non-structural protein (NS) genes from

* Corresponding author. Tel.: +52 57296000x62753; fax: +52 57296000x62806.
E-mail address: hzepeda@ipn.mx (H.M. Zepeda).

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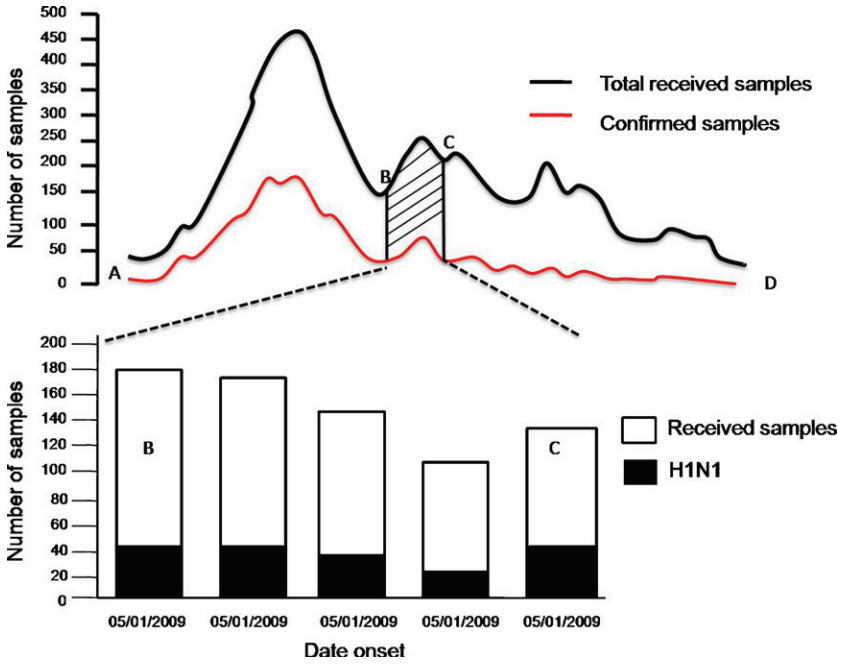

Fig. 1. Number of samples collected during the critical period of the influenza A pH1N1 (2009) outbreak in Mexico City. A total of 751 samples were collected between May 1–5, 2009. The total number of samples received per day is shown in white bars, positive cases of influenza A pH1N1 (2009) are shown in black. Letters shows different days: A = 17th April 2009, B = 1st May 2009, C = 5th May 2009, D = 22nd May 2009.

classic swine influenza A viruses. While the polymerase PB2 (PB2) and polymerase (PA) genes from avian influenza viruses of North American lineage, and the polymerase PB1 (PB1) gene from human seasonal influenza A (H3N2).[4] The genes encoding neuraminidase (NA) and the matrix protein (M) from the latest influenza A viruses circulating in swine populations in Eurasia.[5] At the time of writing this publication five variants of the novel influenza A pH1N1 (2009) had been reported, two of which are current in Mexico.[6] Although Mexico City and California were the first places to report influenza A pH1N1 (2009) cases at the end of March 2009[7,8] and the virus has spread worldwide since, Mexico City has reported the highest number of deaths, most of which occurred within the first few weeks of the pandemic

2. Objectives

The purpose of this study was to characterise genetically the HA and NA genes of the influenza A pandemic (H1N1) 2009 virus during the initial outbreak in Mexico City during the period 1–5 May 2009 in order to monitor for the presence of novel virus variants.

3. Study design

3.1. Sample collection

From May 1–5 a total of 751 samples were collected by throat swabbing of patients who sought medical care at 200 outpatient clinics throughout Mexico City. Samples were frozen at −70 °C until tested. When viral RNA was extracted manually using the RNeasy Mini Kit (Qiagen, Germany) 500-μL of respiratory sample were used, whereas when the MagNA Pure Compact Nucleic Acid Isolation kit I (Roche, Germany) was used, 200 μL were used. In both cases the elution volumes were 50 μL.

3.2. Molecular diagnostics

Molecular analysis of samples were carried out using real-time PCR (RT-PCR) using primers and probes from TIB MOLBIOL (Adelphia, New Jersey, US) as well as those provided by the Centre for Disease Control from the USA (CDC, Atlanta, Georgia, USA). When the primers and probes from TIB MOLBIOL were used, RT-PCR was carried out using the cDNA (synthesized from total RNA with the Transcriptor First Strand cDNA Syntesis Kit; Roche, Germany) and the LightCycler FastStar DNA Master HybProbe (Roche, Germany). Thermal cycling was performed in the LightCycler 2.0 instrument (Roche, Germany) under the following conditions: 10 min at 95 °C; 45 cycles (15 s at 95 °C, 20 s at 58 °C and 25 s at 72 °C), and finally 30 s at 40 °C.

For the CDC protocol, recommendations from the WHO were followed.[11] Primers and probes described in the protocol were used. Real-time PCR was carried out using the Invitrogen SuperScript™III Platinum® One-Step Quantitative Kit (Invitrogen, USA) with 5 μL of total RNA in a 20 μL total reaction volume. Thermal cycling was carried out in a LightCycler 2.0 instrument (Roche, Germany) under the following conditions: 30 min at 50 °C; 2 min at 95 °C; 45 cycles (15 s at 95 °C, 30 s at 55 °C) and 30 s at 40 °C.

Real-time PCR for the diagnosis of influenza A pH1N1 (2009) was carried out using the primers InfAF (5′-AAG ACC AAT CCT GTC ACC TCT GA-3′) and InfAR (5′-CAA AGC GTC TAC GCT GCA GTC C-3′) and the TaqMan probe (5′-TTT GTG TTC ACG CTC ACC GT-3′), labelled at the 5′-end with 6-carboxyfluorescein (FAM) and at the 3′-end with a quencher, designed and performance tested for quantitative real-time PCR assays for influenza A pH1N1 (2009) swine (WHO recommendations)[9] by TIB MOLBIOL (Manheim, Germany).

The identification of H1 was carried out using primers H1SWS (5′-CAT TTG AAA GGT TTG AGA TAT TCC C-3′) and H1SWAs1 (5′-GGA CAT GCT GCC GTT ACA CC-3′) and the TaqMan H1SWP (5′-ACA AGT TCA TGG CCC AAT CAT GAC TCG-3′), labelled at the 5′-end with FAM and at the 3′-end with a quencher (TIB MOLBIOL) tested

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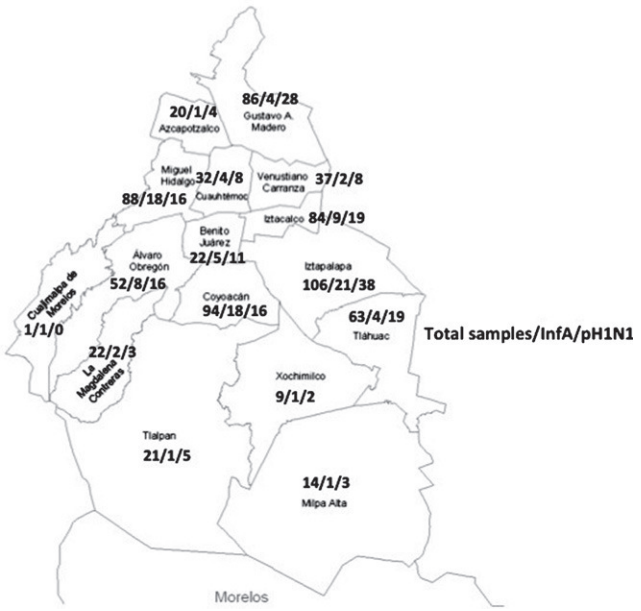

Fig. 2. Number of positive cases for influenza A pH1N1 (2009) in each borough of Mexico City during the outbreak period of May 1–5, 2009. Positive cases of influenza A pH1N1 (2009) reported in 16 boroughs of Mexico City. The North and East zones were the most affected, where the boroughs of Iztapalapa, Gustavo A. Madero, Tlahuac and Iztacalco reported the highest number of cases.

on clinical samples at the University of Bonn, Marburg, Regensburg and also Berlin (RKI) and Hamburg (BNI), according to RKI recommendations.[10]

3.3. Sequencing

The HA and NA genes were sampled randomly and sequenced. Briefly, to amplify a 580-bp fragment from the HA gene (1108–1665 bp; 370–555 Aa), specific primers for the new variant of the influenza A pH1N1 (2009) virus were used (manuscript in progress). To amplify the 616-bp from NA gene (532–1094 pb; 178–365 Aa), the primers recommended by the WHO were used.[11]

The nucleotide sequences of the PCR products were determined using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) and analysed on the ABI Prism 3100 Analyzer Sequencer (Applied Biosystems). The sequences obtained were aligned and analysed using Clustal W[12] and MEGA 4.0.[13]

4. Results

A total of 203 out of the 751 (27%) patients sampled were positive for influenza A pH1N1 (2009) (Fig. 1), where 47% were female and 53% were male. The age of patients positive for influenza A pH1N1 (2009) ranged from 2 months to 81 years old, but the 0–12-year-old group was the most affected (41.8%). Spatial distribution of positive cases showed that 15 of the 16 boroughs in Mexico City had at least one case during the study period. Most of the cases were from the east and north parts of the city, which are the most densely populated areas (east: 15,794 per sq. km; north: 12,226 per sq. km) (Fig. 2). The most common symptoms among the infected subjects were fever over 39 °C (100%), cough (94%), headache (84%), sore throat (72%), rinorrhea (71%) and myalgia (69%), chills (50%), nasal congestion (44%), and conjunctivitis (40%). It is important to highlight that in Mexico City diarrheic cases counted only for 1.3% of the total, whilst USA and Canada reported 25% and 15%, respectively.[14] Partial sequences of NA genes from 63 patients and HA genes from 101 patients were obtained by PCR and sequenced (GeneBank accession numbers HA: GQ292941–GQ293041; NA: GQ292878–GQ292940). Sequence analysis showed five new variants for NA (G249E (GQ292900), M269I (GQ292892), Y274H (GQ292913), T332A (GQ292933), N344K (GQ292882)) and four variants for HA (N461K (GQ293006), K505R (GQ292989), I435V (GQ292995), I527N (GQ292997)). In one patient, variants for HA (N461K) and NA (T331A) were found but no correlation could be established between these and the clinical symptoms and the outcome, which was favorable. The variant G248E was detected on May 2 in one patient in Mexico, and then was detected four and 8 days later among the first cases of influenza A pH1N1 (2009) in Thailand (GeneBank GQ179932 and GQ179931). Thailand health and sanitary authorities and the WHO reported that both patients had visited Mexico in early May.[15] The other three variants for NA (M268I, Y273H, N343K) were detected in one patient each. All patients who were infected with the different variants were treated with oseltamivir immediately after the detection of the virus and recovered completely after 4 or 5 days. Four deaths occurred in Mexico City during our study period, but none could be related to the new mutations in the NA and HA genes. The importance of mapping mutation in the NA and HA genes relies on the fact that this changes could be cumulative and allow the development of resistance to antiviral drugs.[16]

5. Discussion

In this study we described a large number of cases during the epidemic outbreak of influenza A pH1N1(2009) in Mexico City, the importance of describing this specific period of the outbreak relies on the fact that it was approximately then when, according to the local authorities, the epicurve reached the second highest peak[17] (Fig. 1), and the basic reproductive number (R_0)[18,19] reported by the local health authorities reached 1.27[17]; furthermore, during this period governmental sanitary actions were taken, such as the introduction of social distancing measures as recommended by WHO, as well as information campaigns and most importantly, the standardization of sample collection and methods for diagnostic testing, which proved to be efficient in monitoring and therefore diminishing the propagation of the virus among the population. Furthermore, sequencing data showed that, Mexico City could have played an important role for the dissemination of some variants throughout the world as indicated by the match between the Mexican and Thai variant.[15] On the other hand, we have found that the most affected age group was the 0–12 years old, which contrasts with previous reports from USA where the age group most affected was the 10–18 years old,[7] but is consistent with clinical observations from INDRE Mexico and other countries by WHO.[14,20] Sequence analysis from the HA and NA genes showed more variants than those previously reported.[6] Receptor-binding specificity of human and avian viruses are determined by the amino acid residues in the HA receptor-binding pocket, principally amino acids at positions 190 and 225. A single change, HA-Asp 225 to HA-Gly 255, confers binding preference to α 2–6-linked sialic acid over a dual α 2–3/α 2–6 specificity arising from the D222G mutation.[21,22] The functionality of HA cleavage sites is essential for viral infectivity. Although the entire genome from the viruses should ideally be sequenced, the partial HA and NA sequences generated by our group are of importance as they provide a baseline of HA and NA sequences from where the pandemic emerged. The sequences analyzed by our group allowed us to identify non-synonymous changes in four positions in the carboxyl terminal of the haemagglutinin. Of particular importance are the N461K and I527N changes, which could modify the structure. These changes would potentially alter the biological function of the protein. In the NA gene, non-synonymous changes accumulat-

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ing adjacent to mutations that confer resistance to oseltamivir and zanamivir could contribute to the development of further resistance to these drugs.[23–25] Recently, the WHO has reported that an HA mutation D255G has been described in Norway and France and correlated with fatalities. The fatalities we have reported here cannot be correlated to the HA D255G mutation as that region of the HA gene was not sequenced. The regular monitoring of novel variants of influenza A pH1N1 (2009) virus during the actual pandemic, principally those arising in Mexico City could provide important information to predict the emergence of new pathogenic influenza virus resistant to drugs or an increased R_0.

Conflict of interest

The authors declare they do not have conflict of interest.

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